Elections/Restrictions

Applicant thanks the Examiner for reconsidering Applicant's request to search all DNA-based assays together. Applicant's acknowledge the Examiner's indication that claims 1-18, 26 and 33-50 are examined in this application and that claims 19-25 and 27-32 are withdrawn from consideration.

Specification

The Examiner has objected to the disclosure because of missing serial numbers at pages 8 and 9 of the specification. Applicant has amended the specification at pages 8 and 9 to add the appropriate serial numbers. In light of this amendment, Application requests that the objection to the specification be withdrawn.

Sequence Listing

In response to the Examiner's request, Applicants submit a new Sequence Listing.

Applicant has amended the specification to identify the Seq. ID. NOs and replace the original sequence listing with a sequence listing that complies with the sequence rules, 37 C.F.R. §§ 1.821 - 1.825.

The undersigned hereby states that the content of the attached papers and the computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same.

A copy of the *Notice to Comply* is attached hereto as required by United States Patent Office rules governing gene sequences.

Claim Objections

The Examiner has requested that GUS be defined in claim 14. Applicant has defined GUS in claim 14 and 47 as "beta-glucoronidase" as requested by the Examiner. "GUS" is a well known abbreviation for the beta-glucoronidase encoding E. coli uida gene. In light of this Amendment to claim 14, Applicant requests that the objection to claim 14 be withdrawn.

Claims 5 and 38

The Examiner states that claims 5 and 38 lack antecedent basis for "pathway gene". Claims 1 and 33 have been amended to recite a "promoter of a pathway gene" for clarity. This amendment merely made explicit what was implicit in the claim and was not made for a substantial reason related to patentability. As such, claims 5 and 38 have proper antecedent basis for "pathway gene".

Claims 13 and 46

The Examiner states that there is insufficient antecedent basis for "promoter" in claims 13 and 46. As indicated above, claims 1 and 33 have been amended to recite "a promoter of a pathway gene" for clarity. This amendment merely made explicit what was implicit in the claim and was not made for a substantial reason related to patentability. As such, claims 13 and 46 have proper antecedent basis for "promoter".

Claims 15 and 48

The Examiner states that it is unclear what metabolites are encompassed by the term "high-value" in claims 15 and 48. The Examiner's attention is respectfully directed to page 5, lines 9-10 of the application as filed wherein applicants provide:

> The term "high-value secondary metabolites" refers to those secondary metabolites that have valuable commercial applications.

Applicant asserts that in light of this definition it is quite clear what is meant by "high-value". One of ordinary skill in the art understands that "high-value" metabolites are those metabolites that have valuable commercial applications.

In light of the above, Applicants request withdrawal of the claim rejections under 35 U.S.C. § 112, second paragraph.

Applicant respectfully asserts that this application is now in condition for allowance.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned **"VERSION WITH MARKING TO SHOW CHANGES MADE."**

In the event that there are any questions concerning this amendment or the application in general, the Examiner is respectfully urged to telephone the undersigned representative so that prosecution may be expedited.

In the unlikely event that the Patent Office determines that an extension and/or other relief is required as a result of this statement, Applicants petition for any required relief including extensions of time and authorize the Assistant Commissioner to charge the cost of such petitions and/or other fees due to our **Deposit account no. 03-1952** referenced Docket No. **514442001200**. However, the Assistant Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Respectfully submitted,

Dated: January 15, 2002

By: Michael R Ward

Michael R. Ward
Registration No. 38,651

Morrison & Foerster LLP
425 Market Street
San Francisco, California 94105-2482
Telephone: (415) 268-6237
Facsimile: (415) 268-7522

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Replacement Pages

Attached find are pages 8, 9, 18 and 22 with markings to show changes made.

Sequence Listing

Attached is a new Sequence Listing.

In the Claims

1. (Once amended) A method of determining whether a member of a pool of test transcription factor polynucleotides encodes a pathway transcription factor, the method comprising introducing into a cell a nucleic acid comprising a promoter of a pathway gene [promoter] operably linked to a reporter gene and a pool of nucleic acid members comprising test transcription factor polynucleotides and detecting expression from said [pathway gene] promoter in the cell, thereby determining whether a member of the test transcription factor polynucleotide pool encodes a pathway transcription factor.

13. (Once amended) The method of claim 1, wherein said promoter [is] operably linked to a reporter gene is transiently transfected into a cell.

14. (Once amended) The method of claim 1, wherein said reporter gene is beta-glucoronidase (GUS).

33. (Once amended) A method of determining whether two or more members of a pool of test transcription factor polynucleotides are required for expression from a pathway gene promoter, the method comprising introducing into a cell a nucleic acid comprising a promoter of a pathway gene [promoter] operably linked to a reporter gene and a pool of nucleic acid members comprising test transcription factor polynucleotides and detecting expression from said biosynthetic pathway gene promoter in the cell, thereby determining whether two or more members of the test transcription factor polynucleotide pool are required for expression from said [pathway] promoter.

46. (Once amended) The method of claim 33, wherein said promoter [is] operably linked to a reporter gene is transiently transfected into a cell.

47. (Once amended) The method of claim 46, wherein said reporter gene is beta-glucoronidase (GUS).

sorghum, currant, avocado, citrus fruits such as oranges, lemons, grapefruit and tangerines, artichoke, cherries, nuts such as the walnut and peanut, endive, leek, roots, such as arrowroot, beet, cassava, turnip, radish, yam, and sweet potato; and beans. The homologous sequences may also be derived from woody species, such pine, poplar, yew and eucalyptus.

The following description focuses on identification of transcription factors acting on metabolite pathway genes. However, one of skill in art will readily recognize that the methods of the invention can also be applied to genes including, but not limited to, those described above.

Secondary Metabolites of the Invention

The method of this invention identifies one or more transcription factors which increase the expression level of secondary metabolite genes, the biosynthetic rate of plant secondary metabolites, and/or the level of plant secondary metabolites by any significant percentage, but preferably, at least 10%, at least 20%, at least 50%, at least 100% or 200%, at least 300% or 500%, at least 700% or 1000%. Secondary metabolites to be examined in the method of this invention include, but are not limited to, alkaloid compounds, phenolic compounds (*e.g.*, quinones, lignans and flavonoids), and terpenoid compounds (*e.g.*, monoterpenoids, iridoids, sesquiterpenoids, diterpenoids and triterpenoids). In one embodiment, the secondary metabolite is an alkaloid compound or a terpenoid compound. The alkaloid can be a terpenoid indole alkaloid, an indole alkaloid, nicotine, morphine, capsaicin, caffeine, quinine, *etc.* Preferably, the terpenoid is a monoterpene, sesquiterpene, or a diterpene. Pathway genes of secondary metabolites suitable for screening can be identified by scanning published literature on secondary metabolite-producing plants to identify genes whose sequence and expression profile is known.

It will be readily recognized by one of skill in the art that the particular plant secondary metabolite gene examined in the method of this invention is not critical. In one embodiment, endogenous terpenoid pathway genes of *Mentha*, tobacco, and *Taxus* are examined. Peppermint accumulates essential oil (1-2% dw) that consists almost exclusively of monoterpenes, such as menthol and menthone. The first committed step into the pathway is the synthesis of the cyclic molecule limonene. The limonene synthase gene is expressed in leucoplasts of trichome secretory cells, and its expression coincides with the expression of other genes in the pathway. The promoter for the limonene synthase gene was identified and sequenced as described in US Patent Application Serial No. 09/699,083, entitled "Method for Selecting Metabolite Producing Cells", filed October 27, 2000.

Tobacco produces sesquiterpene phytoalexins in response to fungal elicitors. The main sesquiterpene produced is capsidiol. The elicitor-induced accumulation of capsidiol correlates with the induction of 5-epi-aristolochene synthase, which is considered the branch point into sesquiterpene phytoalexin production in tobacco, *eas* genes constitute a 12-15 member strong gene family in tobacco. The promoter of one of the gene members, *eas4*, has been characterized in detail.

Expression of *eas4* (and activity of its promoter) matches closely 5-epi-aristolochene synthase activity and fairly closely capsidiol accumulation in elicited tobacco cell suspension cultures.

Certain *Taxus* species accumulate paclitaxel, which consists of a diterpene moiety and a benzoyl phenylisoserine moiety. Taxadiene synthase catalyzes the first committed step into biosynthesis of the terpenoid moiety of the paclitaxel molecule. The fact that paclitaxel production does not significantly increase when cell suspension cultures are supplemented with phenylalanine, a precursor of the phenylpropanoid moiety, suggests that this pathway is not limiting to Paclitaxel accumulation. In contrast, addition of jasmonate, which induces enzymes of the diterpenoid pathway, greatly increases paclitaxel accumulation in cell culture. This suggests that synthesis and modification of the taxane ring is limiting to paclitaxel accumulation. Taxadiene synthase catalyzes the first step into the taxane biosynthesis pathway. The gene is jasmonate-inducible, and its induction correlates with the onset of paclitaxel accumulation. The promoter for the taxadiene synthase gene was identified and sequenced as described in US Patent Application Serial No. 09/699,083, entitled "Method for Selecting Metabolite Producing Cells", filed October 27, 2000.

Plant Cell Tissue Culture

The method of this invention may be performed in *in vitro* plant cell cultures.

In one embodiment, plant cultures used in the method of this invention are from *Arabidopsis*. Advantageously, *Arabidopsis* is an extremely well developed model system and furthermore, the complete genome is available. Alternatively, cultures can be from any species of plant which expresses high-value secondary metabolites. Preferably, the cultures are from plants that accumulate secondary metabolites in cell culture.

Suspension plant cultures that produce high-value terpenoids include *Piqueria trinervia*, a member of the Asteraceae family, which produces monoterpenes in response to elicitors; *Tobacco*, which produces the sesquiterpene capsidiol in response to fungal elicitors; *Cotton*, which produces sesquiterpene derivatives, such as sesquiterpene aldehyde gossypol in response to fungal elicitors; *Rice,* which accumulates diterpene phytoalexins, such as momilactone and a number of oryzalexins; *Gingko biloba*, which produces diterpenes such as gingkolide and bilobalides, and *Taxus* species, which produce a variety of taxoids. If desired, the method of this invention may also be conducted in other plant species which may produce high-value secondary metabolites under certain conditions.

Callus or cell cultures are obtained, when possible, from academic laboratories and public collections. Alternatively, published protocols may be followed to establish in-house cell cultures for the different species. Typically, explants provide a source of callus that can be used to inoculate liquid cultures. After several transfers and selection for small aggregates, cell cultures can then be scaled up in order to obtain the desired volumes needed for screening and *Agrobacterium* infection. Cell cultures are maintained according to basic protocols described in Evans *et al.*,

fragment was purified away from the 35S promoter fragment. The HindIII/NotI insert fragment from p528 was ligated to this vector fragment, producing plasmid p514.

- Cloning of the limonene synthase (LS) promoter into p512

The LS promoter was PCR-amplified using primers:

O21558: GACCCAAGCTTGTTTGTTTTGACTAAGTTTGGGGGTGAG (SEQ ID NO: 9) and

O21559: ACGCGGATCCGTAGAGAGGCAGTGAAACTACTGAAATTACG (SEQ ID NO: 10).

The same strategy as above was used to clone the LS promoter into pBluescript KS to produce p539. A HindIII/NotI fragment from p539 that contains the promoter was cloned into p512 as above, to generate plasmid p516.

- Transformation of *Agrobacterium* cells with reporter constructs

Cells of nopaline *Agrobacterium* strain ABI were electroporated with binary vectors containing int-GUS fusion constructs. Transformed bacteria were selected on LB plates containing kanamycin (75mg/l), spectinomycin (100mg/l) and chloramphenicol (20mg/l)

- Infiltration of tobacco leaves using *Agrobacterial* cell suspensions

  - Bacterial growth

Agrobacterium cells were re-streaked onto selection plates a few days before infiltration. Overnight cultures were inoculated with *Agrobacterium* cells from these plates into 1ml liquid selection media in deep-well 96-well plates. 85ul of the overnight culture was added to 850ul LB medium supplemented with 10mM MES and 20uM acetosyringone. The resulting culture was grown overnight to saturation (OD ~ 4). 450ul of each transcription factor strain culture were combined to form pools of 4 transcription factor *Agrobacterium* strains. *Agrobacterium* pools were harvested by centrifugation (1500g) and resuspended in 500ul of an infiltration solution containing 10mM MgCl2, 10mM MES and 150uM acetosyringone, where they were incubated for a minimum of 2 hours at room temperature before infiltration. Each cell suspension was adjusted to an OD of 1. Reporter construct -containing strains were grown separately: an overnight 5ml culture was used to inoculate a 50ml culture, which was grown to saturation. Each strain was then resuspended in infiltration solution to a final OD of 1.

  - Infiltration

Promoter intGUS cell pools were produced by combining an equal volume of cell suspensions containing the limonene synthase and taxadiene synthase constructs. TF pools were mixed with an equal volume of promoter-intGUS pools. 100-300 ul of the mixture was infiltrated, into leaves of *Nicotiana benthamiana* plants, using a 1ml syringe. Control suspensions were made up for one half of the reporter construct mix and, for the

CBF3 coding sequence under control of the CaMV 35*S* promoter, was transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation (Koncz et al. (1986) *Mol. Gen. Gen.* 204: 383). *Arabidopsis* plants were transformed with plasmid pMPS13 or the transformation vector pGA643 using the floral dip method (Clough and Bent, (1998) *Plant J.* 16, 735-743). Transformed plants were selected on the basis of kanamycin resistance. Homozygous T3 or T4 plants were used in all experiments.

p511, the RD29A-intGUS construct, was prepared as follows. RD29A and intGUS PCR fragments were cloned in tandem into the vector pMEN65. The plasmid pMEN65 was restricted with the enzymes *HindIII* and *BamHI*, excising a fragment containing the 35S promoter. The main vector fragment was purified by gel electrophoresis. The RD29A and intGUS fragments were generated by the polymerase chain reaction (PCR). RD29A was amplified from 20ng of *A. thaliana* genomic DNA in a 50μL reaction with PFU Turbo DNA polymerase using the primers:

GCCCAAGCTTGGTTGCTATGGTAGGGACTAT (SEQ ID NO: 11) and

TTTGATCCATGGTCCAAAGATTTTTTTCTTTCCA (SEQ ID NO: 12).

The PCR product was purified with a Qiaquick PCR purification column, restricted with the enzymes *HindIII* and *NcoI*, and again purified with a Qiaquick PCR purification column. The intGUS sequence was amplified from 1 ng of the plasmid DNA pEGAD in a 50μL reaction with PFU Turbo DNA polymerase using the primers

AGCGCCATGGCCGGAACCGTCGAGCATGGTCCGTCCTGTAG (SEQ ID NO: 13) and

CGCGGATCCGCCAGGAGAGTTGTTGATTCATTGTTTGC (SEQ ID NO: 14).

The PCR product was purified with a Qiaquick PCR purification column, restricted with the enzymes *NcoI* and *BamHI*, and again purified with a Qiaquick PCR purification column.

The three fragments were ligated together with a molar ratio of 1:2:2 (pMEN65:RD29A:intGUS) using T4 DNA ligase. The RD29A promoter will ligate upstream of the open reading frame of the intGUS gene. The ligation reaction was transformed into the *E. coli* DH5α and plasmid DNAs were isolated from resulting clones. Plasmid DNAs were sequenced across the *HindIII* and *BamHI* sites and through the RD29A and intGUS fragments to ensure that no mutations were introduced by PCR.

Example 6: Increased Production of Metabolites in Plants Overexpressing CBF3

After observing that transient transformation of the transcription factor CBF3 caused 12-fold activation of GUS expression from the rd29a:GUS construct, stable transformants were established and metabolite production levels were determined.

Lyophilized *Arabidopsis* leaf material (30 mg) was extracted with 3 ml deionized water at 80°C for 15 min. The samples were shaken for approximately 1 hour at room temperature and then allowed to stand overnight at 4°C. The extracts were filtered through glass wool and